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Genetics

Deletions within a defective Suppressor-mutator element in maize affect the frequency and developmental timing of its excision from the bronze locus

[transposable elements/changes in state/Enhancer-Inhibitor (*En-I*)/*Zea mays*]

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ABSTRACT **Six independent derivatives of the *bz-m13* allele, which contains a 2.2-kilobase-pair defective Suppressor-mutator (*dSpm*) insertion at the bronze (*bz*) locus, have been isolated and analyzed. The derivatives were selected for alterations in the frequency and timing of somatic reversion; such derivatives have previously been analyzed genetically and designated "changes in state" by McClintock [McClintock, B. (1955) *Carnegie Inst. Washington, Yearb. 54*, 245–255]. All of the derivatives analyzed in the present study revert substantially later in development than the original insertion mutation and some show a very low frequency of reversion as well. All of the derivatives contain insertions at the same site as the parent *bz-m13* allele. Deletions of 400–1300 base pairs were found in the *dSpm* elements in four of the six derivatives; the remaining derivatives could not be distinguished structurally from the original mutant allele. The results suggest that changes in the frequency and developmental timing of excision are attributable to alterations in the *dSpm* element. Furthermore, these data suggest that DNA sequences near the ends of the element are important for responding to the two *trans*-acting functions supplied by the transposition-competent Suppressor-mutator (*Spm*) element.**

Transposable elements were first identified and described in maize (1, 2). Early work revealed the presence of distinct families of elements, each composed of members able to transpose on their own (transposition-competent elements) and members unable to transpose in the absence of a transposition-competent member (defective elements; for reviews see refs. 3 and 4). Recent molecular analyses of maize transposable elements have shown that the defective elements of a family are often deletion derivatives of the transposition-competent elements (5–7).

Mutations caused by the insertion of transposition-competent elements in or near a locus are unstable, reverting both somatically and germinally at a high frequency. Mutations caused by insertions of defective elements are unstable only when a transposition-competent element of the same family is present in the genome. A characteristic of each unstable mutation is its particular pattern of somatic reversion. McClintock described the isolation of derivatives of insertion mutations that showed heritable changes in the frequency and developmental timing of somatic reversion. She designated the new unstable alleles that arose in this manner "changes in state" (8, 9). Since then, derivatives of this kind have been identified by a number of investigators from a variety of insertion mutations (10–16). In addition to changes in the frequency and timing of somatic reversion, these derivatives can involve alterations in the basal level of expression of the affected gene (9, 10, 16) and differences in the rate of germinal change (10, 12, 15).

Two main hypotheses have been proposed to explain the molecular basis for changes in state of unstable mutations. The composition hypothesis (2, 17) postulates that these derivatives are due to changes within the element, whereas the position hypothesis (12) maintains that the element is unaltered but resides in a different location within the affected gene.

A family of maize elements that has been studied in great genetic detail is the Suppressor-mutator system (*Spm*; ref. 18), also called Enhancer-Inhibitor (*En-I*; ref. 19). The two systems are homologous at the genetic level (20) and the DNA level (7). An unstable allele of the *Spm* system at the bronze gene (*bz*) locus in maize has previously been described (15). This allele, termed *bz-m13*, is caused by the insertion of a defective *Spm* element (*dSpm*), which we designate *dSpm-13*. The *Bz* allele is the structural gene for UDP-glucose:flavonol glucosyltransferase; this enzyme catalyzes a late step in anthocyanin pigment biosynthesis (21, 22). A nonfunctional allele at the locus results in a light brown (bronze)-colored aleurone. In the absence of a transposition-competent *Spm* element, a reduced level of UDP-glucose:flavonol glucosyltransferase activity is present in *bz-m13* kernels, but a normal level of anthocyanin pigmentation is seen in the aleurone (Fig. 1*b*; ref. 23). In the presence of *Spm*, a few large sectors of aleurone pigmentation are superimposed on a bronze background (Fig. 1*a*). This response of *bz-m13* to *Spm* is a result of the two *trans*-acting functions of *Spm*: suppression and mutation. Expression of the *bz* locus is "suppressed," resulting in bronze-colored cells; darkly pigmented sectors are due to reversion events induced by the "mutator" function of *Spm* relatively early in endosperm development. In addition to these somatic events, *bz-m13* in the presence of *Spm* leads to many different germinal changes at the *bz* locus. Fully pigmented revertants (Bz′), stable recessive derivatives (bz′), and new unstable derivatives (changes in state) have all been identified (15).

In this study, we characterized six independent unstable derivatives of *bz-m13* that show changes in the frequency and timing of somatic reversion. We first demonstrated that the unique phenotype characteristic of each derivative was due to an alteration linked to the *bz* locus rather than a change in the activity of the *Spm* element. The structure of the *bz* locus in these derivatives was then analyzed by genomic blot hybridization experiments. We show that several derivatives have deletions within the *dSpm-13* element of different sizes and locations. We find no evidence that the position of the element within the locus has changed in any of the derivatives

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Abbreviations: *Spm*, Suppressor-mutator; *dSpm*, defective Suppressor-mutator; *bz*, bronze gene; bp, base pair(s); kb, kilobase pair(s).

nor have we found evidence of other changes in the structure of the locus.

MATERIALS AND METHODS

Plant Materials. The original stock *c-m5 Sh Bz wx-m8/c-m5 Sh Bz wx-m8* from which *bz-m13* was isolated was obtained from B. McClintock. For this reason, her terminology for the system (*Spm*) is used in this paper. The isolation of the *bz-m13* allele and the revertant allele *Bz'-3* has been described (15). In this paper, *bz-m13* refers to the original state of *bz-m13-R5*.

The change in state derivatives were isolated from a cross of *Sh bz-m13/sh bz* in the presence of a standard, active *Spm* onto a *sh bz/sh bz* tester stock. Nonshrunken kernels with an altered pattern of somatic reversion were selected from ears and subsequently crossed onto a *sh bz/sh bz* line with no active *Spm*, to remove *Spm* from the genome. The pattern of somatic reversion of each derivative of *bz-m13* was again tested by reintroducing a standard, active *Spm* from a *sh bz/sh bz* tester stock. Twelve different unstable derivatives of *bz-m13* were originally isolated and designated *bz-m13CS1, bz-m13CS2, bz-m13CS3*, etc. Six of these derivatives (abbreviated as *CS1, CS3, CS5, CS6, CS9*, and *CS12*) were used in this study. To quantify the number and size of revertant sectors in the aleurone of these derivatives, a 6.25-mm^2 section of the crown was analyzed from typical kernels from a set of 1983 crosses of a *sh bz/sh bz* line with a standard active *Spm* onto *Sh bz-m13* (or *Sh CS-*)*/sh bz*.

Genomic Cloning. High molecular weight genomic maize DNA was isolated from immature tassels of plants homozygous for the specified *bz* alleles in the absence of *Spm* by using the method described by Shure *et al.* (24). The genomic cloning of the *bz-m13* allele was described elsewhere (25). DNA isolated from the revertant *Bz'-3* was partially digested with *Bgl* II and cloned into the *Bam*HI site of the λEMBL3 vector (26). Plaque hybridization of the resulting recombinant phage was done as described by Benton and Davis (27), using the plasmid pMBzP22 as the hybridization probe. pMBzP22 contains a unique 2.2-kilobase-pair (kb) DNA fragment from a transcribed region of a nonmutant *Bz* strain (28). Two recombinant phage having strong homology to the *bz* locus probe were isolated. DNA isolated from each phage contained a 7-kb *Bgl* II fragment encompassing *bz* locus DNA. A 650-base-pair (bp) *Sst* I/*Mlu* I fragment from one of these clones was isolated by electroelution from a 1.2% agarose gel, digested with mung-bean nuclease (Pharmacia) to create blunt ends, and subcloned into the *Sma* I site of the pUC8 vector (29). This plasmid, pD3MS9, was subsequently used as the probe in the blotting experiments.

Genomic Blot Hybridization Analysis. Approximately 5 μg of maize genomic DNA was digested for 3 hr with 10–30 units of restriction enzyme under conditions specified by the suppliers (Bethesda Research Laboratories, New England Biolabs, Promega Biotec, Madison, WI). The reaction mixture was then size-fractionated on 0.5–1.1% agarose gels in 50 mM Tris borate/1 mM EDTA, pH 8.1, at 50–60 V for 30–50 hr at 4°C. After denaturation and neutralization of the gel, DNA fragments were transferred to nitrocellulose by overnight blotting (30). Conditions for hybridization, washing, and autoradiography of the filters were essentially as described by Fedoroff *et al.* (31); the plasmid pD3MS9 was labeled with ^{32}P-labeled nucleotides (Amersham) by nick-translation to a specific activity of $6–8 \times 10^8$ cpm/μg.

RESULTS

The *bz-m13* allele, in the presence of *Spm*, gives rise to a variety of derivatives at the *bz* locus. New unstable *bz* alleles (changes in state), similar in phenotype to those reported here, arise at a frequency approaching 1% (unpublished data). The phenotypes of kernels of *bz-m13* and the six unstable derivatives analyzed in this study are shown in Fig. 1 in the presence of *Spm*. In Table 1, we have recorded the average number and size of the revertant sectors present in these alleles. Clearly, the derivatives display marked changes in the frequency and timing of reversion (excision) events. In particular, each exhibits smaller revertant sectors than *bz-m13*, indicating that the excision events occur later in kernel development. However, in the absence of *Spm*, all of these derivatives display a phenotype similar to *bz-m13*: the entire aleurone exhibits normal pigmentation.

The altered pattern of somatic reversion identified in these derivatives could have been due to changes in the *dSpm* element at the *bz* locus or to a change in the transposition-competent *Spm* located elsewhere in the genome. Therefore, we removed the initial *Spm* element from the genome of each presumed derivative allele by meiotic segregation. Reintroducing a standard, active *Spm* into the genome restored the unique kernel phenotype initially observed, indicating that the heritable alteration was linked to the *bz* locus.

A restriction endonuclease map of the *bz-m13* allele had previously been determined from a genomic clone of the mutant (25). A 2.2-kb insertion (the *dSpm-13* element) is present at the *bz* locus in *bz-m13*. Based on restriction enzyme mapping and hybridization studies, this insertion appears to be identical to the *Spm-I8* element isolated from the *wx-m8* allele by Schwarz-Sommer *et al.* (32). These 2.2-kb *dSpm* elements are structurally related to the transposition-competent 8.4-kb *Spm* element (7), with homology to ≈0.9 kb at one end and ≈1.3 kb at the other end of the *Spm* element (unpublished data). We have cloned a stable revertant of *bz-m13*, called *Bz'-3* (Fig. 1*c*), which lacks the 2.2-kb insert (Fig. 2). The 650-bp *Sst* I/*Mlu* I fragment from *Bz'-3* spanning the *dSpm-13* insertion site was subcloned and used as a probe in genomic blot hybridization experiments rather than DNA from *dSpm-13* because the maize genome contains many copies of sequences hybridizing with *dSpm* elements (32).

We began a structural analysis of these derivatives with the assumption that each still contained a *dSpm* element at the *bz* locus (because of the *Spm*-induced instability) but no other major alterations at the locus (because revertant sectors

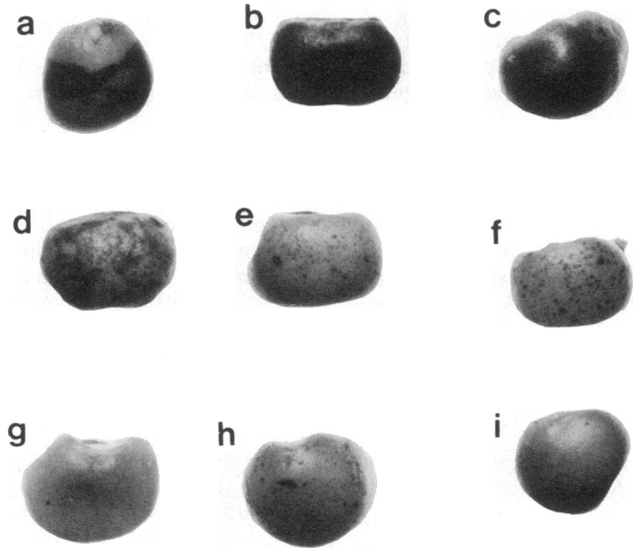

FIG. 1. Aleurone pigmentation in typical kernels from the *bz* alleles analyzed in this study. Genotypes: (*a*) *bz-m13/bz/bz + Spm*; (*b*) *bz-m13/bz-m13/bz-m13 − Spm*; (*c*) *Bz'-3/bz/bz − Spm*; (*d*) *CS1/bz/bz + Spm*; (*e*) *CS3/bz/bz + Spm*; (*f*) *CS5/bz/bz + Spm*; (*g*) *CS6/bz/bz + Spm*; (*h*) *CS9/bz/bz + Spm*; (*i*) *CS12/bz/bz + Spm*.

Table 1. Analysis of revertant sectors in the aleurone of kernels from *bz-m13* and six derivatives

Allele*	Revertant sectors, no. per mm²†	Cells, estimated no. per revertant sector‡
bz-m13	<1	>10³
CS1	21	5–50
CS3	12	5–25
CS5	10	5–25
CS6	<1	5–25
CS9	11	5–25
CS12	<1	5–25

*Typical variegated kernels were evaluated with one copy of the specified allele in the endosperm (*bz-m13/bz/bz* or *CS-/bz/bz*) in the presence of *Spm*.

†The mean number of revertant sectors from the crown of 6–10 kernels of each allele was determined. *CS6* and *CS12* have less than 10 revertant sectors over the entire aleurone.

‡Typical revertant sectors from each allele are reported. However, *CS1* displayed occasional revertant sectors of 100–200 cells, and *CS3*, *CS5*, and *CS9* exhibited occasional revertant sectors of 50–100 cells.

arise). As a first approach, genomic DNAs were digested with *Eco*RI, since no sites for this enzyme are present within the *dSpm-13* element. As shown in Fig. 3*a*, four of the six altered states display a hybridizing *Eco*RI fragment smaller than the 8.2-kb fragment present in *bz-m13*. A second blotting experiment using the enzyme *Bgl* II produced similar results (data not shown). These differences could be explained by a number of hypotheses: a deletion of *dSpm-13* DNA, a rearrangement of sequences including the enzyme sites, or a change in the position of *dSpm-13* in addition to a deletion.

A single *Sph* I site is present within the *dSpm-13* element; therefore, an *Eco*RI/*Sph* I double digestion of *bz-m13* DNA produces hybridizable fragments of 5.1 kb and 3.1 kb. The results from such a digestion with the DNAs from each derivative (Fig. 3*b*) show that the *dSpm* elements from the three derivatives that had the greatest differences from *bz-m13* in the *Eco*RI digest (*CS5*, *CS9*, and *CS12*) did not contain an *Sph* I site, indicating that deletions of *dSpm-13* DNA had occurred. The *dSpm* element in one derivative of *bz-m13 (CS3)* apparently has an *Sph* I site but contains a deletion of ≈400 bp in the smaller *Eco*RI/*Sph* I fragment. Genomic DNA from the other two derivatives (*CS1* and *CS6*) produced two hybridizing fragments of similar size to those of *bz-m13*, indicating that no major change in the size or the position of the *dSpm-13* element had occurred.

Evidence that the position of the *dSpm-13* element had not changed in any of the derivatives was obtained from *Eco*RI/*Hinc*II and *Hinc*II/*Bst*EII double digestions. As shown in Fig. 2, there are two *Hinc*II sites near the ends of the *dSpm-13* element in the *bz-m13* allele. If the changes in the different derivatives were confined to the sequence between these two *Hinc*II sites in the *dSpm-13* element, then

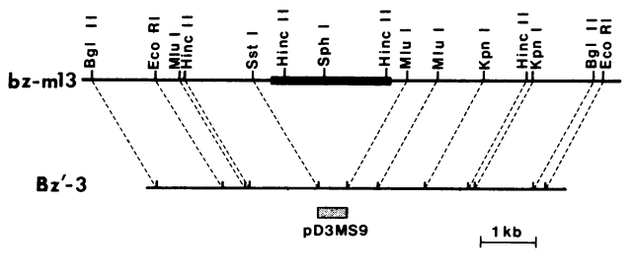

FIG. 2. Restriction endonuclease cleavage site maps of the *bz-m13* and *Bz'-3* alleles. The heavy bar represents the 2.2-kb *dSpm-13* element. Also shown is the *Sst* I/*Mlu* I fragment subcloned in pD3MS9.

the 1.9-kb and 2.6-kb fragments produced by *Hinc*II digestion of DNA from *bz-m13* should also be detected in the derivatives. Although the *Hinc*II digestions were not complete, Fig. 3*c* shows that *CS1*, *CS5*, *CS6*, and *CS12* produce two hybridizable fragments of 2.6 kb and 1.9 kb. *CS3* has the 2.6-kb fragment but not the 1.9-kb fragment in common with *bz-m13*, indicating that the *Hinc*II site on the right end of the element remains but the site on the left is absent or its position is altered. The *Eco*RI/*Hinc*II-digested DNA from *CS9* produced no hybridizable fragment of 1.9 kb and two fragments close to 2.6 kb. A *Bst*EII site is present at the *bz* locus ≈280 bp from the right end of the *dSpm-13* element in *bz-m13*. Therefore, a hybridizable *Hinc*II/*Bst*EII fragment of 380 bp is expected from the DNA of *bz-m13* and all derivatives if no position change occurred. The results from a *Hinc*II/*Bst*EII blotting experiment revealed a hybridizable fragment of the appropriate size for all derivatives (data not shown). We conclude that any change in the position of the *dSpm* in these derivatives would have to be less than ≈50 bp for it to be undetected in these experiments.

The results of additional mapping experiments supported these conclusions and provided further information. Analysis of *Bgl* I digests provided estimates for the sizes of the largest deletions (*CS5*, 1300 bp; *CS9*, 1300 bp; and *CS12*, 1200 bp), and *Aha* III/*Eco*RI, *Acc* I/*Eco*RI, and *Xba* I/*Eco*RI digests of DNA from these derivatives further defined the parts of the *dSpm-13* element affected by these three deletions (data not shown). Data from all genomic blotting experiments were consistent with each other and with the restriction maps shown in Fig. 4. In no instance did we find evidence for a change in the position of the *dSpm* elements or a change in the DNA flanking the elements.

Using these same techniques, no structural alterations have been detected in the *dSpm-13* element from different *bz-m13* reference alleles that display the same pattern of somatic reversion as the *bz-m13* allele used in this study (unpublished data). This indicates that alterations in the *dSpm-13* element occur infrequently, and suggests a correlation between the structure of this element and the pattern of somatic reversion.

DISCUSSION

These experimental results suggest a molecular basis for the occurrence of derivatives of *bz-m13* that display alterations in the frequency and developmental timing of somatic reversion. Four of the six derivatives of *bz-m13* possess deletions of 400–1300 bp that map to internal regions of the *dSpm-13* element. Within the limits of resolution of these experiments, no change in the position of the element within the *bz* locus was detected in any of the derivatives. Therefore, these data support the hypothesis that these derivatives are a result of changes in the structure rather than the position of the inserted element.

Previous evidence that derivatives of this type (changes in state) are due to alterations in the composition of the element at the affected locus has come from analysis of revertants of the Dissociation (*Ds*) mutant *sh-m5933*. Courage-Tebbe *et al.* (33) characterized nine Activator (*Ac*)-induced revertants of this shrunken (*sh*) allele and reported that the only one that produced an altered frequency of chromosome breakage events during endosperm development contained a 2-kb deletion in the *Ds* element. No direct evidence has been obtained supporting the position hypothesis for changes in state, although a number of genetic tests have suggested that the same element may give rise to different patterns of somatic reversion at different locations (12, 34, 35).

Although the suppressor and mutator components of *Spm* have been defined genetically, the basis of their action at the molecular level is not understood. The derivatives analyzed

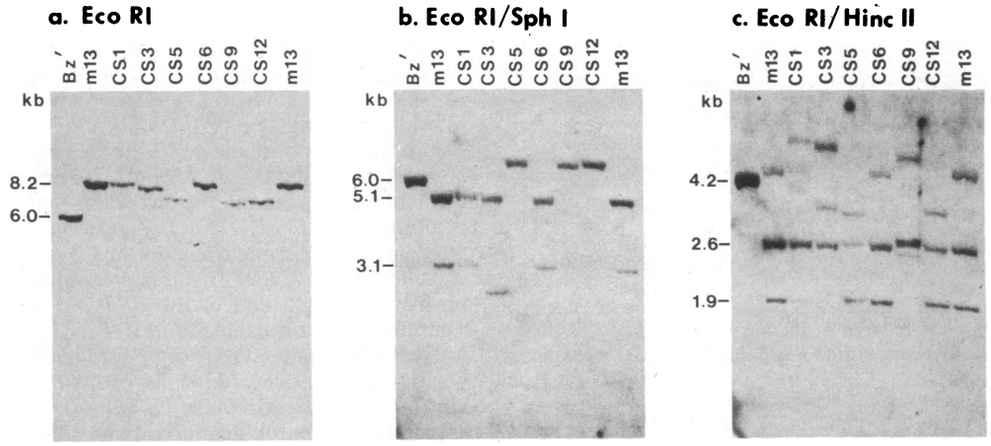

FIG. 3. Blot hybridization analysis of genomic maize DNAs digested with *Eco*RI (*a*), *Eco*RI/*Sph* I (*b*), and *Eco*RI/*Hin*cII (*c*). DNAs were isolated from plants homozygous for the indicated *bz* alleles in the absence of *Spm*; Bz' refers to *Bz'-3* and m13 refers to *bz-m13*. After digestion, DNAs were fractionated on agarose gels, denatured, and transferred to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled pD3MS9, which contains a 650-bp *Sst* I/*Mlu* I fragment of unique sequence maize DNA from *Bz'-3*. The amount of DNA loaded in each lane was not identical. Size standards from λ DNA were included in each gel. Each of these three blotting experiments was repeated with similar results.

in this study differ from the parent *bz-m13* allele by their response to the mutator (excision) function of *Spm*. Therefore, we hoped that a molecular analysis of the *bz* locus in these derivatives would enable us to identify regions of DNA important for this response. A comparison of the results shown in Fig. 4 with the kernel phenotypes in Fig. 1 and Table 1 reveals no obvious correlation between the size or location of the deletions and the pattern of somatic reversion in the kernels. Furthermore, there is no single region of *dSpm-13* DNA that is affected by each of the major deletions from *CS3*, *CS5*, *CS9*, and *CS12*. However, each of these deletions results in the loss of some of the *dSpm-13* DNA near one of the two ends of the element. Molecular analyses of transposable elements from other systems indicates that DNA sequences at the immediate termini are important for transposition (36). A complex series of direct and inverted repeated sequences have been identified within 200 bp of the ends of the *dSpm* elements from *wx-m8* (37) and *bz-m13* (unpublished data). These highly structured sequences at the ends may be important for determining the excision properties of the element; if so, the derivatives analyzed in this study may have arisen by eliminating some of the DNA in these sequences.

The *dSpm* element in each derivative analyzed in this study retained the ability of the parent *dSpm-13* element to respond to the suppressor component of *Spm*, as evidenced by the lack of background pigmentation in the kernels shown in Fig. 1 *d–i*. The results shown in Fig. 4 indicate that the only parts of the *dSpm-13* element present in every derivative are the DNA sequences near each end of the element. Therefore, these data suggest that sequences within ≈150 bp from the end(s) are involved in the response of *dSpm* elements to the suppressor action of *Spm*. Coupled with the previous suggestions concerning the mutator activity, this implies that small regions of DNA near the ends of the *dSpm*-13 element are important for responding to both *Spm* functions. An

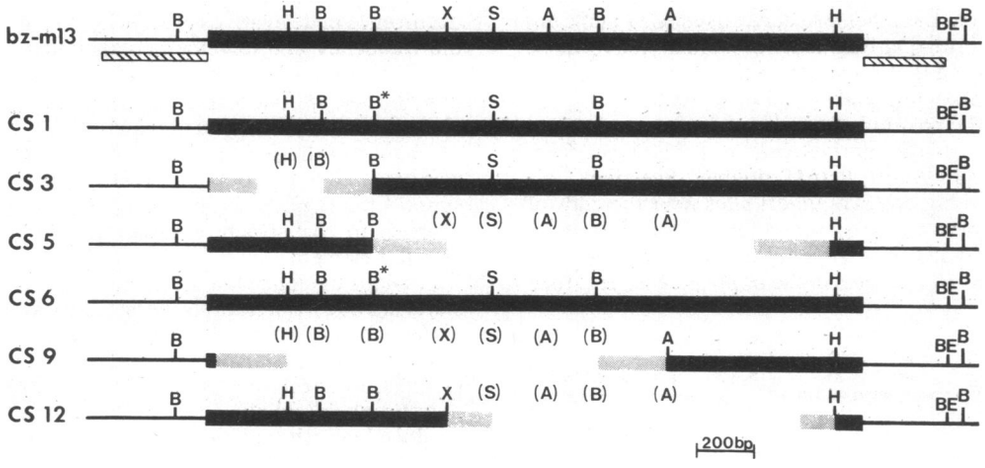

FIG. 4. Restriction endonuclease cleavage site maps of *bz-m13* and six derivatives. These maps indicate the regions of the 2.2-kb *dSpm-13* element determined to be present (solid bars) and absent (open areas) in each of the derivatives by using pD3MS9 (shown in diagonally striped bars) as the hybridization probe. The light shaded bars in *CS3*, *CS5*, *CS9*, and *CS12* represent regions of the *dSpm* elements where uncertainty exists over the exact deletion end points, as determined by the size estimates for each deletion and the restriction sites affected. Restriction enzyme symbols in parentheses indicate that the enzyme was tested on the allele, but no site was present. The absence of a restriction enzyme symbol at a particular site indicates that the enzyme was not tested on the allele. The left-most *Hin*cII site is also an *Acc* I site, so results from the *Acc* I/*Eco*RI blotting experiment mentioned in the text showed that this site is absent in *CS9* and present in *CS5* and *CS12*. The asterisk above the *Bgl* I site in *CS1* and *CS6* indicates that the presence or absence of this site was impossible to determine with the probe used. Restriction sites: A, *Aha* III; B, *Bgl* I; *BE*, BstEII; H, *Hin*cII; S, *Sph* I; X, *Xba* I.

alternative possibility is that the DNA sequence required by the suppressor function is repeated more than once within the *dSpm-13* element. Thus, each derivative, though deleted for a large portion of DNA, may still retain at least one copy of this sequence. We consider this possibility less likely, since our computer analysis of the sequence of the *Spm-18* element (37), which is so far identical to the *dSpm-13* element, shows no appreciable repetitive sequence present in the proper locations.

Analysis of the derivatives with major deletions (*CS3, CS5, CS9*, and *CS12*) also demonstrates that *dSpm* elements can be of a variety of sizes, ranging from the original 2.2-kb element to elements less than 1 kb. In addition, the various *dSpm* elements are heterogeneous with respect to DNA composition, since all four of these deletions are different from one another. These findings are analogous to those from the *Ac-Ds* transposable element system of maize, where the defective element of this system, *Ds*, varies greatly in size and composition (6).

Two derivatives of *bz-m13* (*CS1* and *CS6*) are intriguing because they produce hybridizable restriction fragments indistinguishable in size from those produced by *bz-m13* in every genomic blotting experiment conducted. We suspect that the insertions in these derivatives differ from *dSpm-13* at the DNA level by a small deletion(s), a critical base change(s), or a small rearrangement(s) that was not detectable by our present analysis. It is also possible that the altered pattern of somatic reversion in each of these is due to a change in position of *dSpm-13* of less than ≈50 bp or the presence of a hypothetical *Spm* modifying factor linked to the *bz* locus. The derivatives containing detectable deletions within the *dSpm-13* element may also contain additional changes such as those described above. DNA sequence analyses of these derivatives should resolve these uncertainties.

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